

Development and Validation of HPLC Method for the Quantification of Anticancer Peptide

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Abstract: As the prevalence of cancer persists, the development of new and more advanced therapies remains a main concern in the health research. In this work the development and validation of HPLC method for the quantification of anticancer peptide were checked for analytical validation parameters were accuracy, precision (repeatability and reproducibility), specificity, sensitivity (limit of detection and limit of quantification) and linearity. The accuracy and precision of the method have been tested and the coefficients of variation have been found to be less than 2% according to ICH validation guideline. The linearity range was found to be 5-35 $\mu\text{g/mL}$, LOD-5.65 $\mu\text{g/mL}$ and LOQ-17.1322 $\mu\text{g/mL}$ respectively. In conclusion, a new RP-HPLC method has been developed and validated successfully for the quantitative analysis for the peptide in this study.

Keywords: Cancer, RP-HPLC, Quantitative analysis, Peptide, Analytical method

Introduction

High performance liquid chromatography (HPLC) has developed into a widely used, well-established tool for the analysis and refinement of biomolecules. The rationale for the central role that HPLC now plays in analyzing and refining proteins / peptides is resolution. HPLC is able to split polypeptides of nearly identical sequences, not only for small peptides such as those obtained through trypsin digestion, but even for much larger proteins. In recent years, significant concentration has been paid to the use of peptides as therapeutic agents and this inclination is expected to continue. Because of their wide-ranging activities in many physiological processes such as acting as signaling molecules and growth factors, peptides play a considerable role as therapeutic mediators, chiefly in the areas of neurology, endocrinology and hematology. Even though peptides have been shown to be promising therapeutics for many disease conditions, developing them as drugs is a challenging process since the accurate quantification of peptides is one of these challenges and their poor stability, losses during manufacturing and delivery process and their high cost are other issues. Incorrect quantification of peptides may lead to false conclusions in research and may also adversely affect the dosage that is prescribed for patients.

Still nevertheless, discovery of protein and tumor-related peptides and proteins is expected to create a “new wave” of more effective and selective anticancer drugs in the future, capturing the large share of the cancer therapeutic market. It may play a vital role in next generation cancer therapy. And the peptide *p*-ACC1 designed for tumor suppressor by interaction with BRCT domains¹. The present work is focused to develop the analytical method and validation of HPLC for quantification of the peptide

Experimental

The HPLC system of SHIMADZU - UFLC series with Rhodyne injector 77251, Hamilton syringe-25 μ L C18G120A column, 250x4.6 mm 5U with guard column WM03-0.22, detector UV at 220 nm. The chromatographic analyses were carried out at 5 $^{\circ}$ C using reversed phase. The peptide was purchased from life technologies. Spectroscopic grade trifluoroacetic acid (TFA) and HPLC grade acetonitrile were purchased from Sigma-Aldrich, USA

Methods

The mobile phase A 1% w/v of trifluoro acetic acid in water and Mobile phase B 0.1% w/v of trifluoro acetic acid in 80:20 ratio of acetonitrile and water were used. The flow rate was set at 1.0 mL/min and the wavelength set as 220 nm. The injection volume was kept as 20 μ L and the sample solution was prepared by accurately weighed quantity of peptide sample 5 mg in 5 mL volumetric flask and dissolved with sufficient water and made the volume up to 5 mL ultrapure water.

The analytical validation parameters were selected according to the Validation of analytical procedures (Q2, R1) of ICH Harmonised Tripartite Guideline². Analytical validation parameters were accuracy, precision (repeatability and reproducibility) specificity, sensitivity (limit of detection, LOD; limit of quantification, (LOQ) and linearity

Accuracy

Three different concentrations of samples including high, medium and low concentrations were used for determination of accuracy. Accuracy was expressed as the percentage of coefficient of variation (% CV). All values found for the three different concentrations of peptide were lower than 2% (n=3) (Table 1).

Table 1. Accuracy values for three different concentrations of peptide (n=3)

Theoretical Concentration, μ g/mL	Area, mAu	Practical Concentration, μ g/mL	% Recovery
25	567.98	24.87	99.48
25	546.37	24.22	92.88
25	544.13	24.01	96.04
Average		24.15	96.133
% Bias		3.002	
100	983.12	99.81	99.81
100	993.55	99.23	99.23
100	979.21	99.12	99.12
Average		99.38	99.386
% Bias		0.233	
200	1581.503	198.65	99.32
200	1588.627	197.87	98.93
200	1589.888	198.65	99.32
Average		198.39	99.195
% Bias		0.654	

Precision

The precision structure was evaluated in stipulations of repeatability and reproducibility. For injection repeatability, six analyses of one concentration were performed in the same sample. For system repeatability and reproducibility, analyses of six diverse samples of one concentration were performed in the same day and 2 different days (Table 2). All CV values were found to be lower than 2%.

Table 2. Injection repeatability values (n=6)

Theoretical concentration, $\mu\text{g/mL}$	Area mAu	Practical concentration, $\mu\text{g/mL}$	Area mAu	Practical concentration, $\mu\text{g/mL}$
200	1588.627	198.87	1926.66	210.77
200	1589.888	196.56	1996.21	210.66
200	1592.827	189.55	1543.33	189.87
200	1583.432	199.10	1576.55	198.90
200	1599.922	187.90	1675.23	197.78
200	1600.974	191.32	1565.87	196.99
Average	1592.6116	193.883	1713.98	200.828
Theoretical concentration	199.98		200.65	
	SD	8.28646	SD	4.90701
	CV	0.13	CV	0.122

Table 3. Linearity parameters of the analytical method

Parameters	Values
Linearity Range	5-35 $\mu\text{g/mL}$
Slope	15.181
Intercept	-1.506
LOD	5, 65 $\mu\text{g/mL}$
LOQ	17.1322 $\mu\text{g/mL}$

Sensitivity

Sensitivity was calculated based on the residual standard deviation (RSD) of the retort and the slope. Average values of six calibration curves were used for this purpose. By this method LOD was found as 5,65 $\mu\text{g/mL}$ and LOQ was found as 17.1322 $\mu\text{g/mL}$ using the following equations, respectively.

$$\text{LOD} = 4.1 (\text{SD}/\text{Slope}) \text{ Equation 1}$$

$$\text{LOQ} = 10 (\text{SD}/\text{Slope}) \text{ Equation 2}$$

Linearity

The HPLC chromatogram of the peptide could be seen in Figure 1. The retention time (t_R) was 13.99517 min at 220 nm. The Mobile phase A 1% w/v of trifluoro acetic acid in water and mobile phase B 0.1% w/v of trifluoro acetic acid in 80:20 ratio of acetonitrile and water solvent system. The calibration curve was prepared from five sample concentrations within the range of 5-35 $\mu\text{g/mL}$ (Figure 1) and found linear in this range. There were seven series of samples prepared and average values were used to prepare the calibration curve. The r^2 value was found as 0.926 (Figure 2).

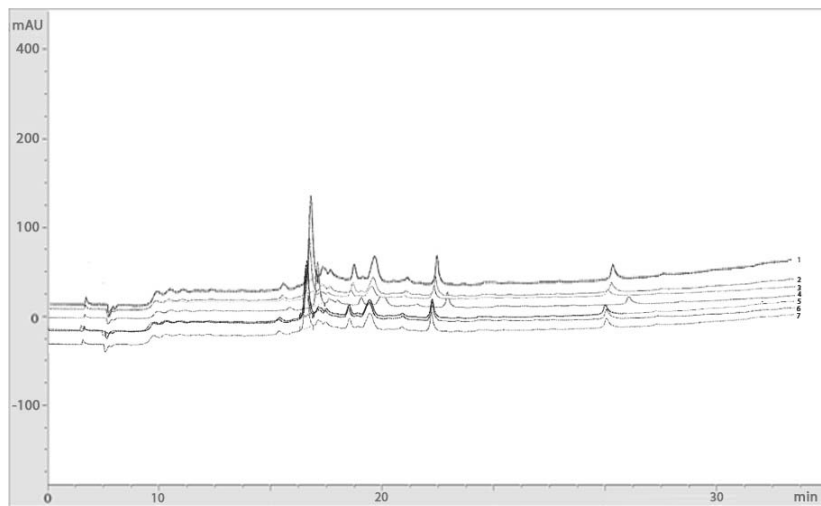


Figure 1. RP-HPLC chromatogram of *p*-ACC1 in different concentrations (5-35 µg/mL)

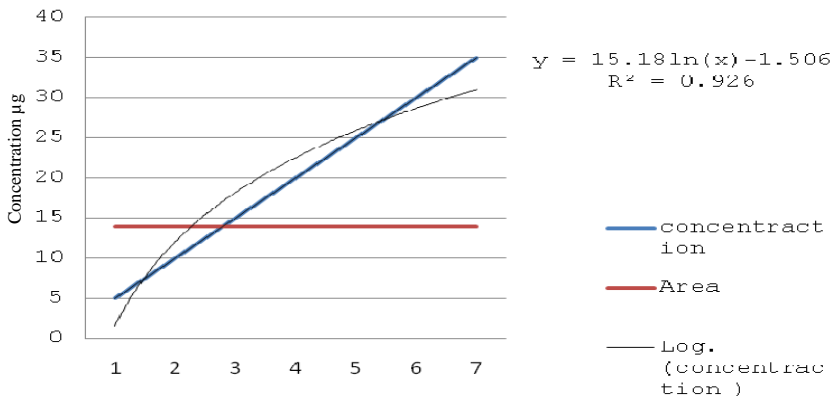
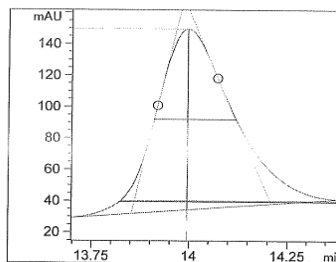


Figure 2. Calibration curves

Peak description [min]:

Signal: DAD1 B, Sig=220,4 Ref=off
 RetTime: 13.99517 k': 4.40354
 Height: 115.15367 Area: 1581.5034
 Start: 13.70362 End: 14.39077
 Skew: 0.38946 Excess: 0.13501
 Width at half height: 0.21111
 5 sigma: 0.57077
 tangent: 0.35762
 tailing: 0.56633
 Symmetry: 0.69387
 USP Tailing: 1.67663
 Integration type: MM
 Time increment [msec]: 400.00000
 Data points: 183



Statistical moments (BB peak detection):

M0: 1576.220569
 M1: 14.022289
 M2: 0.009562
 M3: 0.000364
 M4: 0.000287

Efficiency: Plates per ..

Method	column	meter
Tangent method:	24497	97990
Halfwidth method:	24341	97362
5 sigma method:	15026	60106
Statistical:	20563	82252

Figure 3. Peak descriptions

System suitability

Inject reference solution and ensure column efficiency not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and % of relative standard deviation of replicate injections of reference solution is not more than 2.0.

Results and Discussion

The next generation cancer therapy will be purely depends on the peptides. Within just a few years, the pipeline of therapeutic peptides has grown significantly. In 2004, there were 400 peptides in development globally; almost three times as many as in 2001. While the current pipeline is still primarily driven by early-stage projects (discovery/preclinical stages), an increasing number of peptides are maturing into later stage trials and reaching the market³⁻⁵. The majority of peptides currently in the market are generics with relatively low growth potential due to several inherent limitations like: peptides generally suffer from low stability (quick degradation and clearance from the body); low oral bioavailability (injection required); difficulties in delivery (cross-membrane transportation); risk of immunogenic effects; as well as challenging and costly synthesis (solubility issues) and so on⁶. For overcome the above the issues, we require to formulate the peptide in to targeted drug delivery by itself or conjugation to another drug / drug carrier system⁵. However, there has not been an analytical method reported in the literature for this peptide. Thus, a RP-HPLC method has been developed and validated for the quantitative analysis of *p*-ACC1 in the present study.

Conclusion

The peptide which was taken in to the study has vital function for upcoming therapy of anti cancer chemotherapy. The peptide can be loaded with carrier and antibody labeled drug delivery system. In this study, a suitable RP-HPLC method was successfully developed and validated for the quantitative analysis.

Reference

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